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Population Heterogeneity of *Agrobacterium tumefaciens* in Galls of *Populus* L. from a Single Nursery

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This study focused on the natural crown gall infections occurring in a *Leuce* poplar nursery. Soil effects on crown gall frequency were detected, indicating that contamination was due to a resident *Agrobacterium tumefaciens* population, which was present before seedling plantation. The crown gall frequency on poplar progenies varied from 3 to 67%, indicating the feasibility of improvement in crown gall resistance. Of 129 tumor isolates, 128 were pathogenic. These isolates were of biotype 1 or 2. Biochemical, serological, and antibiotic resistance typing results concurred, indicating the presence of four biotype 1 and two biotype 2 resident subpopulations. No significant change was noticed in the relative proportions of subpopulations from one year to another. Pathogenic subpopulations both in vitro and in planta were susceptible to Kerr K84 (P. B. New and A. Kerr, J. Appl. Bacteriol. 90:172–179, 1972). In addition, no serological cross-reactions were found to occur between K84 and the pathogenic subpopulations.

The improvement of *Populus* L. in France has partly been focused on the selection of species and hybrids belonging to the *Leuce* section (*Populus alba*, *P. tremula*, and *P. tremuloides*). These hybrids are well suited to large-scale planting in hydromorphic forest soils. However, a major bacterial pathogen, *Agrobacterium tumefaciens* (Smith & Townsend) Conn, has recently appeared in progenies of this section, causing severe crown gall attacks in nurseries and plantations. Previously, *A. tumefaciens* had occasionally been isolated from *Leuce* poplar trees (8, 11, 18, 19, 26), but the current infections were found to be so severe that control of crown gall has become a priority for *Leuce* improvement.

Selecting for crown gall-resistant hybrids would be an interesting but long-term means of control. Alternatively, biological control with Kerr *A. radiobacter* K84, which was successfully used to control fruit tree crown gall (22), could be rapidly undertaken. However, the use of K84 failed because the pathogenic strains became resistant to K84 bacteriocin, probably as a result of plasmid exchange between pathogenic and nonpathogenic strains. To avoid the detrimental evolution of this *Agrobacterium* population, we chose to estimate the hazards of increased bacteriocin resistance in pathogenic agrobacteria before extending the use of K84 to forestry purposes.

Characterization of a single *A. tumefaciens* population was a necessary prerequisite for a study of the population dynamics at an infraspecific level. Therefore, we determined the distribution of the galled seedlings in a nursery and the genetic diversity of the strains isolated from these trees.

MATERIALS AND METHODS

The present study was based on observations of galled *Populus* seedlings grown in the same locality: a single nursery plot established on forest soil. The nursery, located at the Forest Trees Research Station of the Institut Nationale de la Recherche Agronomique (INRA) in Orléans, France, was designed to propagate 28 hybrid progenies obtained from controlled crosses. The seedlings received no intentional inoculation. The galls, therefore, originated from naturally field-contaminated plant materials.

Plot design. The nursery plot was composed of 24 blocks where progeny seedlings were planted in an incomplete block design. The total number of seedlings of a given progeny varied from 2 to 140; consequently, a given progeny was met in 1 to 7 blocks according to its number of seedlings. Gall-free seedlings from greenhouse-germinated seeds were planted in July 1984 and harvested 7 months later in February 1985.

Isolates. The 129 isolates studied came from 34 different galled plants, 30 of which were seedlings that belonged to a sample of 17 progenies out of the 25 that had developed galls in the nursery plot (one to three seedlings per progeny). The remaining four galls studied came from seedlings grown at the boundary of the plot and thus belonged to four additional progenies. One gall per plant was used; the galls studied developed close to the crowns. There were 1 to 8 isolates per gall except for one gall from which 11 isolates were obtained.

Isolations were performed with freshly harvested nonnecrotic galls. The supernatants of the crushed gall sections were spread on four selective agar media in petri dishes. Two media were selective for biotype 1 isolates, one with mannitol at 10 g/liter (24) and the other with dulcitol at 2.73 g/liter (4) as the carbon source. The two other media were selective for biotype 2 isolates, both with erythritol at either 3.05 g/liter (4) or 5 g/liter (21) as the carbon source. Genus and biotype determinations were done according to the method of Kerr and Panagopoulos (13), a method modified by using liquid media and a rotary shaker (instead of agar media); this allowed the assessment of results within a week.

The isolates were routinely grown at 27°C on YPGA medium (yeast extract, 5 g/liter; Bacto-Peptone, 5 g/liter glucose, 10 g/liter; Bacto-Agar 15 g/liter; all from Difco Laboratories). Long-term storage was achieved by lyophilization.

Test for pathogenicity. Each isolate was tested for pathogenicity on 5 to 6 leaved tomato seedlings (cultivar Monfavet 63-5) grown in a growth chamber at 22°C, with a 16-h day and

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TABLE 1. Percentage of naturally galled seedlings in hybrid progenies of *Leuce* poplars in a single nursery plot

Progeny code[a]	Breeding origin of the progeny[b]	Serogroups detected[c]	No. of galled seedlings/ progeny	Total no. of seedlings/ progeny	%[d]
738	a × ta		14	21	66.7†
739	a × ta		2	70	2.9*
906	(ta × a) × a	1b, 1c, 2ac	10	28	35.7†
907	(ta × a) × a	1b, 2ac	24	98	24.5†
908	(ta × a) × a	2al	6	73	8.2
909	(ts × a) × a	1b, 1c	19	93	20.4
910	(ts × a) × a	1b	13	140	9.3
914	(ts × a) × a	1a, 1b, 2ac	19	35	54.3†
920	(ts × a) × ta	1b, 1c	12	84	14.3
924	(ts × a) × ta	1a, 1b	10	42	23.8
927	(ts × a) × ta	1a, 1b	7	63	11.1
928	(ta × a) × ta	1a, 1b, 2ac	7	116	6.0*
929	(ta × a) × ta	1a, 1b	4	49	8.2
930	(ta × a) × ta		2	102	2.0*
931	(ts × a) × ta		4	77	5.2*
933	(ta × a) × ta	1b	5	63	7.9
934	(ta × a) × ta	1a, 1b, 2al	7	108	6.5*
935	(ts × a) × ta	1c	28	49	57.1†
936	(ts × a) × ta		4	112	3.6*

[a] Only progenies composed of at least 20 seedlings grown in at least five different blocks inside the nursery were maintained in this part of the study.

[b] Symbols: ta, *P. tremula*; a, *P. alba*; ts, *P. tremuloides*.

[c] Serogroups detected in one-, two- or three-galled seedlings belonging to the progeny.

[d] *, Progeny significantly less infected than the mean percentage of the plot, 14.0%; †, progeny significantly more infected (0.01 level).

high relative humidity. The plants were pricked with a toothpick which had been dipped into a 48-h-old culture on agar medium. Tumor formations were recorded 1 to 3 weeks later. The isolates that did not result in tumor formation on the tomato seedlings were inoculated on cuttings of a *Leuce* hybrid (*P. tremula* × *P. alba*; no. 712-7, INRA, Orléans) in a growth chamber.

Serological survey. Five sera were prepared from five *Agrobacterium* strains. Four strains—2177, 2516, 2517, and 2519—were isolated from *Populus* galls harvested from the nursery studied. The fifth serum was prepared with strain 1929 isolated from the gall of a wild cherry tree (*Prunus avium* L.) from another location. In addition, we prepared a serum from Kerr K84. The strains cited here are registered under these numbers in the *Collection Francaise de Bactéries Phytopathogènes* (INRA, Angers) except strain K84, which is registered under no. 1937.

The antigenic solutions were extracellular materials obtained as previously described (7). Briefly, layers of 48-h-old YPGA-grown isolates were suspended in phosphate-buffered saline (pH 7.2) and agitated for 1 h. After filtration (0.45-μm pore size), the solutions were precipitated with ammonium sulfate (100% saturation) and then dialyzed against tap water for one night. The dialyzed antigenic solutions were spectrophotometrically adjusted to 2 mg/ml (globulin equivalent) and then injected into Fauve de Bourgogne rabbits according to the following schedule: day 1, 1 mg; day 3, 2 mg; days 8, 10, 12, 15, and 19, 4 mg. Serum titers were determined on day 17, and bleedings were done on the following days. The sera were stored at −20°C.

Serum titers varied from 1/2,000 to 1/8,000. A routine serological study was done by indirect immunofluorescence on pure culture isolates (6) with dilutions 1/500 and 1/1,000. Reactions were recorded as positive when the cell walls were fluorescent and negative when the fluorescence was restricted to the flagellum.

MIC pattern determination. The determination of MICs was performed by the agar dilution technique in YPGA medium with an inoculum-replicating apparatus (25). The following 18 antibiotics and inhibitory compounds were assayed: ampicillin, methicillin sodium salt, and kanamycin (Bristol Laboratories) carbenicillin and ticarcillin (Beecham Laboratories); streptomycin (Specia), neomycin and chloramphenicol (Roussel-UCLAF); gentamicin (Unilabo); oxytetracyclin and polymyxin-B sulfate (Pfizer Inc.); erythromycin (Abbott Laboratories); rifamycin sodium salt (Lepetit); novobiocin sodium salt (Theraplix); nalidixic acid (Winthrop Laboratories); cupric sulfate, mercuric chloride, and sodium azide (Prolabo). The range of serial concentrations tested varied from 0.5 to 128 ppm of active matter. About 10^4 48-h-old cells were deposited per inoculation spot. After 48 h, cell growth was recorded as positive when at least 10 colonies per inoculation spot could be observed.

Biological control. In vitro susceptibility tests to agrocin 84 and greenhouse experiments of K84 biological control were done according to the method of Kerr and Panagopoulos (13).

Numerical analysis. Principal coordinate analysis was performed with an Apple IIe (Cupertino, CA-95014) microcomputer with a program adapted from that of Lebart et al. (16) by G. Lachaud. Variance analysis of frequencies was done according to the method of Lellouch and Lazar (17).

RESULTS

Distribution of the galled seedlings. Of the 1,568 seedlings growing in the nursery plot, 219 (14%) had tumors on their roots and crowns. The plot was divided into 24 blocks with six progenies per block. Since the number of seedlings per progeny varied from 2 to 140, the analysis of effect for progenies was performed only with progenies composed of at least 20 seedlings, which came from at least five different blocks. Because of this incomplete design, block effect was not considered in this part of the study. The frequency of crown gall infections varied from 3 to 67% (Table 1), indicating a significant effect (0.01 level) for progenies. In a second analysis, block effect was estimated regardless of the progeny and found to be significant at the 0.01 level. Some blocks had a higher or lower percentage of galled seedlings than the 14% average of the total plot (Table 2). In a third analysis, we did not find a significantly higher number of susceptible progenies in the blocks with the highest percentage, nor more resistant progenies in poorly attacked blocks.

TABLE 2. Frequency of galled seedlings within blocks of the nursery plot[a]

No. of galled seedlings/total no. (*P*)[b]		
10/73 (ND)	10/81 (ND)	6/71 (ND)
14/27 (>0.001)	9/65 (ND)	5/76 (<0.05)
6/65 (ND)	16/69 (>0.05)	4/59 (ND)
19/98 (ND)	6/54 (ND)	5/52 (ND)
14/55 (>0.05)	14/72 (ND)	3/56 (<0.05)
9/39 (ND)	6/55 (ND)	13/75 (ND)
4/95 (<0.001)	13/70 (ND)	6/56 (ND)
8/70 (ND)	11/77 (ND)	8/58 (ND)

[a] Seedlings of six progenies were planted in each block. Ratios indicate the number of seedlings, galled and total, received regardless of the progeny.

[b] Levels of significance of galled-seedling frequency of block compared to the 14% frequency average of the plot are indicated in parentheses: ND, no difference (0.05 level); <0.001 and <0.05, lower than average; >0.001 and >0.05, greater than average.

Characterization of population diversity. All except one of the 129 isolates were pathogenic on tomato seedlings, with a tumorization delay that varied from 11 to more than 21 days. The single isolate that was found to be nontumorigenic on tomato plants induced no true tumor after 1 month on *Populus* 712-7 cuttings.

Biochemical studies indicated that all isolates were of either biotype 1 (79%) or biotype 2 (21%). Four biotype 2 isolates induced an alkaline instead of an acid reaction with litmus milk. Exhaustive serological investigations indicated the presence of six serogroups: 1a, 1b, 1c, 1d, 2ac, and 2al (Table 3). The two serogroups observed with biotype 2 isolates were the groups previously described as acidifying and alkalinizing the litmus milk, i.e., 2ac and 2al, respectively. Serogroup 1d was composed of the single isolate, which induced no tumor grown on tomato and poplar seedlings.

Several sera were prepared from agrobacteria arbitrarily chosen from isolates of Orléans poplar galls. Every serum led to one of the serological patterns presented in Table 3. Only one serum per serogroup was maintained: i.e., anti-2177, anti-2516, and anti-2519. By contrast, since a group of isolates gave no cross-reaction with any sera, we arbitrarily chose one nonreacting isolate (no. 2517) to elaborate a new serum, which positively detected the so-called 1c serogroup. According to this serogrouping, strains 2177, 2516, 2517, and 2519 were grouped as serotypes 1a, 1b, 1c, and 2ac, respectively. Isolates 2518 and 2520 were considered to be types 1d and 2al, respectively.

In addition, there was no cross-reaction of any isolate, except for one, with serum 1937 prepared from strain K84. Moreover, K84 did not cross-react with any serum prepared from the strains that belonged to this nursery; the cross-reaction titers were always less than 1/200.

From the collection of 129 isolates, 17 of the 18 inhibitory compounds assayed induced various MICs and could be used to identify the isolates. Methicillin gave identical MICs with every isolate. To further analyze the relation between MIC patterns, we calculated a matrix of variance and covariance based on MIC logarithms. The data were centered around their mean but were not reduced by variance. In this manner, a greater discriminating power was given to the most variable MICs. Using the matrix, we performed a principal coordinate analysis. A plot of the MIC patterns along the first two coordinates (Fig. 1) placed the isolates in distinct groups. These antibiogroups fitted with the serogroups described previously. As indicated in Table 4, MIC differences between groups 1a and 1b were observed with polymyxin B, chloramphenicol, and, with less discriminating power, kanamycin. These differences were ascertained by the antibiotic diffusion technique by using susceptibility disks. Although it was harder to discriminate clearly the 1b or 1c isolates on the basis of MIC patterns, significant differences were observed between the 1b and 1c subpopulations (Fig. 1). By contrast, the nonpathogenic isolate (1d) was readily distinguished by ticarcillin and novobiocin. The

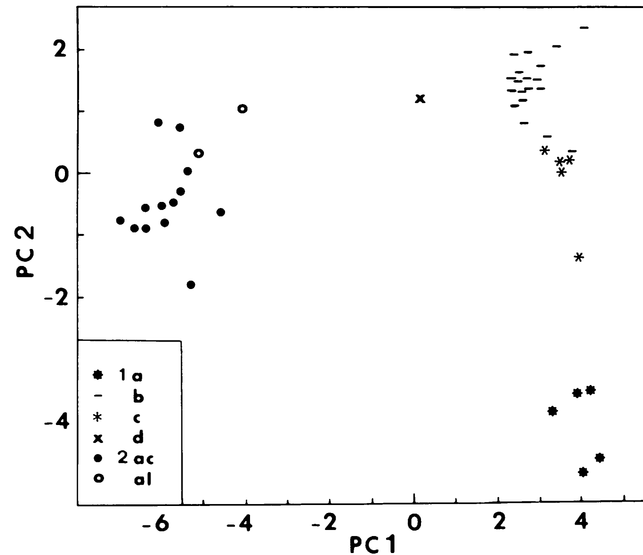

FIG. 1. Distribution of MIC patterns of *A. tumefaciens* on the first two axes (PC1 and PC2) derived from a principal coordinate analysis. As many isolates had identical MIC patterns, one dot frequently represents more than one isolate.

TABLE 3. Serogrouping of *A. tumefaciens* strains isolated from a single poplar nursery

Biotype	Group	Antiserum[a]					
		2177	2516	1929	2517	2519	K84
1	a	+ +	+ +	−	−	−	−
	b	−	+ +	+	−	−	−
	c	−	−	−	+ +	−	−
	d	−	−	−	−	−	−
2	ac	−	−	−	−	+ +	−[b]
	al	−	−	−	−	−	−
	K84	−	−	−	−	−	+ +

[a] Symbols: +, positive immunofluorescence reaction with a 1/500 serum dilution; + +, positive reaction with 1/1,000 serum dilution; −, no immunofluorescence with 1/500 dilution.

[b] Except for one isolate.

TABLE 4. Intrinsic antibiotic resistance patterns of *A. tumefaciens* serogroups

Inhibitory compound	MIC (mg/liter) for serogroup:					
	1a	1b	1c	1d	2ac	2al
Beta-lactams						
Ampicillin	>128	128	>128	128	4–8	4
Methicillin	>128	>128	>128	>128	>128	>128
Carbenicillin	16	16	16	16	8–32	128
Ticarcillin	32	32	32	>128	>128	>128
Aminosides						
Streptomycin	16	16	16	16	1–8	4
Neomycin	16	16	16	32	16	16
Kanamycin	16	32	64	8	8–16	8
Gentamicin	8	8–16	8	8	1–2	2
Miscellaneous						
Oxytetracyclin	1	1–2	1	1	0.5–2	1
Erythromycin	64–128	64–128	64	128	1–8	8
Chloramphenicol	8–16	32–64	32	32	32–64	32
Rifamycin	32	32	32	64	16–32	32
Novobiocin	2	2	1–2	128	4–8	4
Polymyxin B	32–64	1	2	1	1–2	1
Nalidixic acid	>128	>128	>128	8	128	128
Other inhibitory compounds						
Cupric sulfate	250	250	250	250	125	125
Mercuric chloride	16	16–64	16	8	2–4	8
Sodium azide	16	16	16	16	4	4

TABLE 5. Repartition of serogroups among isolates and galls[a]

Serogroup	1985		1986	
	No. of isolates (%)	No. of galls (%)[b]	No. of isolates (%)	No. of galls (%)[b]
1a	17 (13.2)	7 (20.6)	14 (11.6)	10 (14.5)
1b	67 (51.9)	27 (79.4)	64 (53.3)	44 (63.8)
1c	17 (13.2)	6 (17.6)	5 (4.1)	5 (7.2)
1d	1 (0.8)	1 (2.9)	0 (0)	0 (0)
2ac	23 (17.8)	12 (35.3)	38 (31.6)	23 (33.3)
2al	4 (3.1)	2 (5.9)	0 (0)	0 (0)

[a] 1985 isolates were obtained from 1-year-old, galled seedlings grown in the nursery plot. 1986 isolates were obtained from 3-year-old, galled root cuttings grown in the vicinity of the nursery plot.

[b] Galls where at least one isolate of a given serogroup was found. The sum of every serogroup percentage may be greater than 100% because several serogroups could be found on the same gall.

discrimination between the biotype 2 isolates that acidify or alkalinize litmus milk could be assessed with carbenicillin. However, MIC patterns were more variable among biotype 2 than among biotype 1 isolates.

Relative frequencies of subpopulations. The number of isolates per serogroup and the number of galls contaminated by a given serogroup are indicated in Table 5. The data are presented in two distinct samplings: first, with the 129 isolates obtained in 1985 from the 1-year-old, galled seedlings of the present nursery plot; and second, with 120 isolates from galls of 3-year-old root cuttings of *Leuce* poplar cultivars, which were grown in the vicinity of the plot and harvested in 1986. The relative percentages of every serogroup were similar in both samples. As indicated in Table 5, two serogroups were detected in 1985 but not in 1986. However, these groups showed rather low frequencies in 1985 and their absence in the 1986 sample was insignificant. As an indication, when several isolates originated from the same gall, the number of serogroups per gall varied from one to three.

Biological control. Representatives of each pathogenic subgroup (i.e., 1a, 1b, 1c, 2ac, and 2al) were tested for their susceptibility to K84. All representative organisms were susceptible to agrocin 84 in vitro, showing an inhibition zone around the K84 colony, and were biologically controlled when tested on tomato seedlings, developing no gall when coinoculated with K84.

DISCUSSION

Until now, the study of natural populations of *A. tumefaciens* has received little attention. The characterization of agrobacterial diversity has already been done on a large scale with strains originating from different geographical regions (9, 11, 18, 19, 26) but not at the level of a local population. In our study, two kinds of data were obtained from a single poplar nursery: the variability of the frequency of galled seedlings in a plot and the evidence for phenotypic heterogeneity among *Agrobacterium* isolates.

Crown gall frequency. The demonstration of block effect (Table 2) indicates that the topographic location of seedlings was a significant influence in determining whether a plant was infected. This block effect was probably linked with variations of the *A. tumefaciens* population level in the soil. *Agrobacterium* therefore appears to be established in the nursery before seedling plantation and was not randomly carried in with diseased materials. As a consequence, the population we analyzed was indigenous to that forest soil. The fact that a pathogenic *A. tumefaciens* populaton was established in the forest soil is important because *Leuce* hybrids are well suited to forest settlements. Since *Agrobacterium* spp. can live saprophytically for long periods in soils (1), forest soils are potential reservoirs of pathogenic bacteria. On the other hand, we demonstrated here that K84 biological control is feasible and this is currently being tested in the nursery involved here. However, if agrobacteria are common in forest soils, then it would be difficult to extend the practice of the K84 biological control to the forest settlement level.

Numerous reports indicate the frequent occurrence of crown gall on poplar plants that belong to the *Leuce* section, a finding which is contrary to rare observations among *Aigeros* and *Tacamahaca* cultivars (8, 11, 18, 19, 26). The present study gives additional information on the host plant control of *A. tumefaciens*-Leuce poplar compatibility at an infraspecific level. Actually, *Leuce* progenies exposed to natural contamination showed different frequencies of crown gall attack (Table 1). This result seems to indicate the feasibility of selection for crown gall-resistant progenies of *Leuce* poplars. However, the results must be ascertained at the cultivar level by using controlled inoculations with known strains because differential interactions between *Agrobacterium* strains and cultivars have already been shown in various other genera (15, 20, 23).

Characterization of the *Agrobacterium* population. The serology (Table 3) and the intrinsic antibiotic resistance patterns demonstrated here (Fig. 1) enabled us to distinguish a range of subpopulations present in the indigenous population. Antibiotic resistance typing acted in this study as a confirmation of the population structure found by serological investigations. Actually, it has been shown for *Rhizobium* spp., a genus closely related to *Agrobacterium*, that several serological patterns could be found within strains related on the basis of their antibiotic resistance pattern (14); in other cases, the serology was less variable than the intrinsic antibiotic resistance (5). As a consequence, these methods (5, 14) could not be used on their own for strain identification, but they can be useful when used simultaneously to identify *Rhizobium* isolates (3, 10). Therefore, the correspondence observed between agrobacterial antibiotic resistance and serological typings does not necessarily indicate a functional correlation but did show that both methods describe the same phenotypic structures of the local population. As observed with serogroups 1b and 1c (Fig. 1), the application to MIC determination was emphasized by principal coordinate analysis, which allowed the recognition of MIC differences between these subpopulations even when it was rather difficult to distinguish their respective isolates by MIC typing.

The isolates formed a sample of the strains resident in the nursery soil which was skewed in favor of those most compatible to poplar. However, one isolate was unable to form a tumor when inoculated into poplar cuttings. Gall isolation of nonpathogenic or weakly pathogenic strains is common (11). Even so, the methods used in this study showed that this nonpathogenic isolate belonged to a subgroup that is readily distinguishable from the truly pathogenic biotype 1 isolates (Fig. 1).

Of a total of 129 isolates, two main populations (the two biotypes) were divided into four and two subpopulations, respectively. To our knowledge, this is the first time that such heterogeneity has been found in a local population of *Agrobacterium* strains. In comparison, Jenkins and Bottomley (10) found seven different antibiotic resistance patterns

among 300 *Rhizobium* nodule isolates in the same field. This is a quite similar degree of heterogeneity. The techniques we used also showed that one subpopulation was the most common gall occupant bacterium; i.e., 1b isolates were found in 79% of the galls examined. In addition, agrobacteria belonging to different subpopulations were commonly found in the same gall. This finding is comparable to the simultaneous detections of biotypes 1 and 2 in the same gall by Anderson and Moore (2).

In another independent sampling, which was undertaken later in the same nursery but in another plot, the same main serogroups—1a, 1b, 1c, and 2ac—were found in quite similar proportions (Table 5). In this case the galled plants were planted in 1983 instead of 1984 as in the previous sampling. Since the contaminations occurred at the moment of the plantation (the galls studied developed close to the crowns), the two sets of data presented Table 5 indicate that relative proportions of every subpopulation did not significantly evolve from 1 year to another. Although *A. tumefaciens* undergoes intraspecific transformations in the laboratory (12), the extent to which transformations occur in nature is unknown. Are the serogroups the consequence of gentetic mutations of two original strains, one for each biotype, or are they the result of six different sources of contamination?

Because a given subpopulation may have a particular behavior, the occurrence of population heterogeneity of *A. tumefaciens* is relevant in crown gall disease control. We therefore took into consideration the relative diversity within a single agrobacterial population in naturally contaminated soils when sampling isolates, and we then verified the potentiality of K84 biological control on representative isolates of every subgroup (instead of randomly chosen sample of isolates). Furthermore, the implications of introducing a strain such as K84 into the environment should also be considered in terms of population dynamics, looking for the behavior of each subpopulation. For this purpose, serology appears to be a suitable tool that allows the individual recognition of each pathogenic subpopulation as well as strain K84.

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